Function and Structure in Phage Qβ RNA Replicase

ASSOCIATION OF EF-Tu-Ts WITH THE OTHER ENZYME SUBUNITS*

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Qβ replicase is a complex of four nonidentical subunits readily dissociable into two subcomplexes: 30 S ribosomal protein S1 and the phage-coded polypeptide (Subunits I + II) and protein synthesis elongation factors EF-Tu and EF-Ts (Subunits III + IV). The affinity of the two subcomplexes for one another increases with increasing ionic strength. The enzyme is capable of initiation of RNA synthesis with synthetic templates only when in the low ionic strength conformation. Elongation of initiated polynucleotide chains is not affected by ionic strength.

Addition of Qβ RNA to the enzyme also alters its quaternary structure: the EF-Tu-Ts cannot be covalently attached to the other enzyme subunits with bifunctional cross-linking reagents in the presence of RNA. This conformational change is not influenced by ionic strength. The addition of Qβ RNA to the enzyme, does not result in the release of EF-Tu-Ts from the other enzyme subunits: whereas free EF-Tu-Ts binds GDP independently of salt concentration, this binding by Qβ replicase is sensitive to high ionic strength and remains so in the presence of Qβ RNA. Furthermore, RNA does not allow the release of EF-Ts from EF-Tu by GTP as measured by sensitivity of EF-Ts activity to N-ethylmaleimide.

Qβ phage RNA replicase (nucleoside triphosphate:RNA nucleotidyltransferase (RNA dependent)) is composed of four nonidentical subunits, three of which are coded for by the host, Escherichia coli (1, 2). The host polypeptides are part of the protein synthetic machinery in uninfected cells: the largest (Mr = 70,000) has recently been found to be 30 S ribosomal protein S1 (3, 4). The smaller two are protein synthesis elongation factors EF-Tu (5) and EF-Ts (Mr = 45,000 and 35,000, respectively) (5). The phage-coded subunit (Mr = 65,000) must be responsible for the high specificity of the enzyme for its natural template (6), since the other subunits are host-coded and are found in RNA bacteriophage RNA replicases with different template specificities (7). The phage-coded subunit is capable of performing the elongation reaction on synthetic templates in the absence of the host-coded subunits (8). Subunit I is required only for initiation of transcription of Qβ RNA (9), while EF-Tu and EF-Ts are necessary for initiation of transcription of synthetic templates (8) as well as Qβ RNA (10).

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‡ The abbreviations used are: EF-Tu and EF-Ts, protein synthesis elongation factors Tu and Ts, respectively; EF-Tu-Ts, the complex of EF-Tu and EF-Ts; SDS, sodium dodecyl sulfate.
* G. Carmichael, personal communication.

The enzyme is composed of one each of the four subunits (11). It can be further subdivided into two smaller complexes composed of the two larger subunits and EF-Tu-Ts (1, 2, 11). Using the technique of intramolecular protein cross-linking we have recently found that the two smaller complexes are associated in a different, “tighter,” complex at high ionic strength, than at low ionic strength (11). In addition, we have shown that both GDP binding by EF-Tu and [3H]GDP exchange catalyzed by EF-Ts are inhibited by increasing ionic strength when these polypeptides form part of the replicase enzyme, although with the individual factors these activities are not influenced by salt concentration (8). We report here that increasing ionic strength results in a concomitant decrease in the ability of the enzyme to initiate transcription of synthetic templates, but not elongation of initiated RNA molecules.

There is considerable evidence that addition of RNA to Qβ replicase alters the quaternary structure of the enzyme: when either synthetic templates (1, 8) or Qβ RNA (2) are mixed with the enzyme (with or without GTP) and the mixture is subjected to zone sedimentation on sucrose gradients, the EF-Tu and EF-Ts are separated from the other enzyme subunits which remain bound to the RNA. Furthermore we have shown (11) that when Qβ RNA is added to the enzyme the EF-Tu-Ts can no longer be cross-linked to the other enzyme subunits by dimethyl suberimidate. These results suggest the possibility that the presence of RNA results in the release of EF-Tu and EF-Ts from the enzyme. In this paper we present evidence that the conformational change induced by Qβ RNA...
Poly(A,C) by Qp replicase. Replicase (5 µg/ml) was added to standard buffer containing 0.7 mM GTP, 1 mM phosphoenolpyruvate, 0.1 mM dithioerythritol, 20% glycerol, 10 µg/ml of pyruvate kinase, and NaCl to give the ionic strengths shown. Equivalent ionic strengths were determined by measuring conductivity and comparison with the ionic strengths of known NaCl solutions. This mixture was incubated for 5 min at 0°C and then 30 s at 25°C before the addition of poly(A,C) (0.1 mg/ml) to initiate the reaction (time = zero). Samples (0.1 ml) were removed at the times shown and aurintricarboxylic acid (2 x 10⁻⁵ M) was added to prevent further initiations. [3H]UTP (0.2 mM, 50 Ci/mol) was then added and the reactions were incubated for 5 min at 25°C to allow completion of all of the chains which had been initiated. The samples were precipitated, filtered, washed, and counted as described by Kamen (12). Ionic strengths: O, 0.075; △, 0.125; ∆, 0.175; D, 0.25.

Fig. 2 (center). The effect of ionic strength on the elongation is independent of ionic strength and that the elongation factors are not normally released from the other enzyme subunits.

EXPERIMENTAL PROCEDURE

Materials—Dimethyl suberimidate, sodium dodecyl sulfate, dithioerythritol, and N-ethylmaleimide were purchased from Pierce Chemical Co. (Rockford, Ill.). Acrylamide and bisacrylamide were from Bio-Rad Laboratories (Richmond, Calif.). Nucleoside triphosphates and poly(A,C) were from P-L Biochemicals (Milwaukee). [8-³H]GTP, [3H]GDP, and [5,6-³H]UTP were from New England Nuclear (Boston). Aurintricarboxylic acid (Aluminon grade) was from Fisher.

Standard buffer was 0.05 M Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM EDTA, and 0.1 mM dithioerythritol.

Qp replicase was purified to homogeneity from Qp amB86-infected Escherichia coli K12 strain Q13 according to Kamen (12) with modifications described previously (5). The enzyme was judged to be greater than 95% pure as determined by SDS polyacrylamide gel electrophoresis. It was found to be approximately 80% active in initiating transcription of synthetic templates as described previously (8). Qp RNA was purified by the method of Yamamoto et al. (13). Host factor was purified from a high salt ribosomal wash (14, 15). The procedure of Arakawa et al. (16) was followed. EF-Tu and EF-Ts were prepared by the method of Kamen et al. (9) with modifications described by Wahba et al. (5). Qp RNA was purified by the method of Yamamoto et al. (13). Host factor was purified from a high salt ribosomal wash (14, 15). The procedure of Arakawa et al. (16) was followed. EF-Tu and EF-Ts were prepared by the method of Kamen et al. (9) with modifications described by Wahba et al. (5).

RESULTS

Inhibition of Qp Replicase Activity by Salt—In order to determine the effect of ionic strength on the rate of initiation of Qp replicase on synthetic templates, the enzyme was incubated at 25°C with poly(A,C) and GTP (the initiating nucleoside triphosphate (18)) at a variety of salt concentrations. Samples were periodically removed to tubes containing aurintricarboxylic acid, a reagent that prevents Qp replicase initiation but not elongation (19). [3H]GDP was then added and the reaction was incubated for 5 min at 25°C. (This is approximately 3 times as long as required to complete the chains.) The results (Fig. 2) show that there was little, if any, inhibition of elongation with alteration of Qp RNA. The mixtures were incubated for 10 min at 21°C before the addition of 2.6 mM [3H]GDP. After a further 5 min at 21°C, the samples were filtered and counted as described previously (8).

Fig. 3 (right). Effect of salt concentration on the binding of GDP to Qp replicase in the presence and absence of Qp RNA. Qp replicase was then added and the reaction was incubated for 5 min at 25°C. The [3H]GDP was then added and the reaction was incubated for 5 min at 25°C. (This is approximately 3 times as long as required to complete the chains.)

The effect of ionic strength on the rate of elongation was also measured: sample time (5 min at 25°C) for complete initiation on poly(A,C) was allowed at low ionic strength before the addition of NaCl. (Formation of aurintricarboxylic acid-resistant initiation complexes is complete within 1 min at 25°C.)

The [3H]GDP was then added to permit elongation and sequential samples were removed and precipitated. Again initiation was prevented by the addition of aurintricarboxylic acid with the UTP. The results (Fig. 2) show that there was little, if any, effect of ionic strength on the elongation rate.

Correlation of Initiation Inhibition with Alteration of Enzyme Quaternary Structure—Since several recent lines of evidence indicate that the replicase subunits associate differently with varying ionic strength (1, 8, 11), it is important to determine whether the change in initiation activity correlates with the change in enzyme quaternary structure. A convenient assay for this alteration in subunit association is the binding of [3H]GTP to EF-Tu. Although this binding is not normally affected by ionic strength, the binding of [3H]GTP to EF-Tu when it is a subunit of Qp replicase is strongly inhibited by high ionic strength (8). Clearly this inhibition must result from interaction with the other replicase subunits and can be

1 T. Blumenthal, unpublished observations.
assumed to reflect the nature of the association between the polypeptides. Table I shows that, although the binding of [3H]GTP by EF-Tu-Ts is not affected by ionic strength, the binding of [3H]GTP by Qβ replicase and the initiation on poly(A,C) by the enzyme are lost in parallel. Thus, the alteration in enzyme quaternary structure resulting in loss of GTP-binding activity correlates with the loss of RNA synthesis activity with increasing ionic strength.

Effect of ionic strength on dissociation of Qβ replicase

Binding of [3H]GTP by Qβ RNA—The amount of 215,000 molecular weight complex formed by cross-linking the four Qβ replicase subunits together with dimethyl suberimidate in the presence or absence of RNA was measured at varying ionic strengths. It was found (Table II) that, although increased amounts of this complex were formed at higher ionic strengths, approximately the same percentage of the complex was lost in each case when an equimolar quantity of Qβ RNA was added. This suggests that the dissociating effect of RNA is not related to the dissociating effect of low ionic strength.

TABLE I

Effect of ionic strength on GTP binding and initiation of transcription

Initiation activity is estimated from the initial slopes of the curves of Fig. 1. EF-Tu-Ts or Qβ replicase (12 pmol) was assayed for GTP-binding activity as described previously (5, 8) in the standard reaction mixture brought to the ionic strengths shown with NaCl. The [3H]GTP (5.66 Ci/mmol) concentration was 0.4 PM. The 100% values in each case were approximately 1 pmol of GTP bound/pmol of added protein. Further reductions in the ionic strength did not result in increased GTP-binding activity or RNA synthesis (data not shown).

<table>
<thead>
<tr>
<th>Ionic strength</th>
<th>GTP Binding</th>
<th>Initiation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EF-Tu Ts</td>
<td>Qβ replicase</td>
</tr>
<tr>
<td>0.075</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.125</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.175</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.250</td>
<td>95</td>
<td>18</td>
</tr>
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</table>

Discussion

The association of Qβ replicase subunits has been shown to be sensitive to ionic strength: EF-Tu-Ts separates from the complex of Subunits 1 + II during glycerol gradient centrifugation in low ionic strength buffer (1); significant amounts of elongation factors bound to the other replicase subunits after treatment with chemical cross-linkers are found only when treated at high ionic strengths (11). Moreover, the poly(C)-dependent pol(y)G polymerase isolated from phage Qβ-infected Escherichia coli has been shown to be inhibited by increasing salt concentration (21). We demonstrate here that high ionic strength also alters the functioning of Qβ replicase. Initiation of transcription of synthetic templates is inhibited by salt, while elongation of already initiated polynucleotide chains is not. A consideration then, is whether this inability to initiate is related to the type of association of EF-Tu and EF-Ts with the two larger subunits. If it is, we would expect to find the two to be correlated as ionic strength is changed. A convenient assay for subunit association is the binding of GTP to EF-Ts, since this binding is only sensitive to ionic strength when the EF-Tu is a subunit of Qβ replicase (8). This binding is lost in parallel with the loss of initiation activity. We suggest that this correlation of the two activities is a reflection of their common dependence on the tightness of binding of the protein synthesis elongation factors to the other enzyme subunits. These results are reproducible stimulation of the EF-Ts activity by N-ethylmaleimide (Table III), indicating that addition of RNA and host factor does not result in release of EF-Tu-Ts. We cannot explain the small but reproducible stimulation of the EF-Ts activity by N-ethylmaleimide.

<table>
<thead>
<tr>
<th>Ionic strength</th>
<th>Amount 215,000 molecular weight complex formed (arbitrary units) % remaining RNA + RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>41</td>
</tr>
<tr>
<td>0.4</td>
<td>114</td>
</tr>
<tr>
<td>1.0</td>
<td>148</td>
</tr>
</tbody>
</table>

TABLE III

Inhibition of EF-Ts activity by N-Ethylmaleimide

EF-Tu-Ts (5 x 10⁻⁷ M) and Qβ replicase (3 x 10⁻⁴ M) were treated with N-ethylmaleimide (or an equivalent volume of ethanol) in the presence of equimolar Qβ RNA and host factor with and without 20 mM GTP as described under “Experimental Procedure.” After quenching the reactions with dithioerythritol, the preparations were assayed for EF-Ts activity. In this assay the EF-Tu alone bound 192 cpm.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cpm bound (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF-Tu-Ts</td>
<td>2491 573</td>
</tr>
<tr>
<td>N-Ethylmaleimide</td>
<td>2209 (91%) 738 (129%)</td>
</tr>
<tr>
<td>GTP +  N-ethylmaleimide</td>
<td>756 (31%) 777 (136%)</td>
</tr>
</tbody>
</table>
do not answer the question of whether the loss of enzymatic activity is a direct result of the loss of ability of the EF-Tu to bind GTP. The association of EF-Tu and EF-Ts with Subunits I + II is also influenced by the presence of RNA: velocity sedimentation of Qβ RNA after incubation with either Qβ RNA or poly(A,C) (with or without GTP) results in at least partial separation of EF-Tu and EF-Ts from the complex of Subunits I + II + template (2, 8); Qβ RNA and a variety of synthetic RNAs prevent the covalent attachment of the elongation factors to the other subunits by cross-linking reagents (11). Our results indicate that this effect and the effect of ionic strength on subunit association are not related: cross-linking of enzyme subunits into a single large complex was inhibited by Qβ RNA to the same degree at the three ionic strengths tested. This suggested that the apparent dissociating effects of low ionic strength and RNA are different phenomena at the molecular level: that low ionic strength may actually result in the release of the EF-Tu-Ts, while Qβ RNA causes only an alteration in their structure so that either they are no longer close enough to be cross-linked together by dimethyl suberimidate or the amino acids involved in the cross-linking are no longer accessible.

Two tests of this hypothesis indicate that the elongation factors are not actually released from the two larger subunits in the presence of Qβ RNA. When EF-Tu is part of Qβ replicase, Qβ RNA does not free the GDP binding activity from sensitivity to ionic strength. Since binding of GDP to EF-Tu-Ts is not sensitive to salt (8), these results indicate that addition of Qβ RNA does not produce EF-Tu-Ts separated from the other enzyme subunits. The other experiment involves inactivation of EF-Ts activity by N-ethylmaleimide: if addition of Qβ RNA and host factor caused the separation of EF-Tu-Ts from the rest of the enzyme, then GTP would separate the EF-Tu from the EF-Ts, thereby exposing the N-ethylmaleimide-sensitive sulfhydryl group on EF-Ts (20) and resulting in the inactivation of the EF-Ts by N-ethylmaleimide. This is in fact what occurs when Qβ RNA, host factor and GTP are added to EF-Tu-Ts. But when they are added to whole Qβ replicase the EF-Ts activity is slightly stimulated by the N-ethylmaleimide. Thus, again we find that RNA does not release the EF-Tu-Ts. Carmichael et al. (22), using antibodies directed against EF-Tu and EF-Ts, present evidence that these subunits remain associated with the initiated enzyme-RNA complex.

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